EXHIBIT 3

JOURNAL OF VIROLOGY, Feb. 1995, p. 1059–1070
0022-538X/95/$04.00+0

Vol. 69, No. 2

Virus-Induced Immunosuppression: Immune System-Mediated Destruction of Virus-Infected Dendritic Cells Results in Generalized Immune Suppression

PERSEPHONE BORROW,* CLAIRE F. EVANS, AND MICHAEL B. A. OLDSTONE

Department of Neuropharmacology, Division of Virology, The Scripps Research Institute, La Jolla, California 92037

Received 4 August 1994/Accepted 8 November 1994

Despite the clinical importance of virus-induced immunosuppression, how virus infection may lead to a generalized suppression of the host immune response is poorly understood. To elucidate the principles involved, we analyzed the mechanism by which a lymphocytic choriomeningitis virus (LCMV) variant produces a generalized immune suppression in its natural host, the mouse. Whereas adult mice inoculated intravenously with LCMV Armstrong rapidly clear the infection and remain immunocompetent, inoculation with the Armstrong-derived LCMV variant clone 13, which differs from its parent virus at only two amino acid positions, by contrast results in persistent infection and a generalized deficit in responsiveness to subsequent immune challenge. Here we show that the immune suppression induced by LCMV clone 13 is associated with a CD8-dependent loss of interdigitating dendritic cells from periarteriolar lymphoid sheaths in the spleen and, functionally, with a deficit in the ability of splenocytes from infected mice to stimulate the proliferation of naive T cells in a primary mixed lymphocyte reaction. Dendritic cells are not depleted in immunocompetent Armstrong-infected mice. LCMV Armstrong and clone 13 exhibit differences in their tropism within the spleen, with clone 13 causing a higher level of infection of antigen-presenting cells in the white pulp, including periarterial interdigitating dendritic cells, than Armstrong, thereby rendering these cells targets for destruction by the antiviral $CD8^+$ cytotoxic T-lymphocyte response which is induced at early times following infection with either virus. Our findings illustrate the key role that virus tropism may play in determining pathogenicity and, further, document a mechanism for virus-induced immunosuppression which may contribute to the clinically important immune suppression associated with many virus infections, including human immunodeficiency virus type 1.

Virus infection is frequently associated with a transient or more long-lasting generalized suppression of the host immune response. While the induction of immune suppression is potentially advantageous for the virus, being one of the mechanisms by which viruses may escape clearance and establish a persistent infection in vivo (reviewed in references 4 and 47), the clinical consequence for the host may be severe. Examples of virus infections in humans associated with clinically important immune suppression include human immunodeficiency virus type 1 (HIV-1) infection, which results in AIDS, an expanding pandemic that has caused almost 2 million deaths in the last decade (39), and measles virus infection, which results in the death of >1 million children per year (8, 16, 65). Despite its clinical importance, the pathogenesis of virus-induced immunosuppression is still poorly understood (reviewed in references 10, 19, 26, 38, and 41). To gain further insight into the mechanism(s) by which viruses produce immune suppression in their natural hosts, we have studied a murine model of virus-induced immunosuppression: the generalized immune suppression that occurs following intravenous (i.v.) infection of adult mice with certain isolates of lymphocytic choriomeningitis virus (LCMV).

LCMV is an arenavirus that is a natural pathogen of mice. It can cause either acute or persistent infections in vivo. The outcome of infection is dependent on both host (the mouse strain and age of the animal at the time of infection) and viral factors (the virus isolate used and dose and route of inoculation) (reviewed in reference 14). For example, intraperitoneal inoculation of adult mice with LCMV results in an acute infection in which virus is cleared within 7 to 10 days. Virus clearance is mediated by major histocompatibility complex (MHC) class I-restricted $CD8^+$ antiviral cytotoxic T lymphocytes (CTL) (15, 22). By contrast, mice infected neonatally or in utero with LCMV become persistently infected with the virus for life. Persistent infection occurs because the animals are unable to mount an effective antiviral CTL response primarily because of thymic deletion of LCMV-reactive T cells (28, 52). As anticipated, the CTL deficit in mice infected in utero or at birth is LCMV specific and such mice can make CTL and antibody immune responses to other antigens (49, 64). Mice can also be persistently infected with LCMV as adults by i.v. inoculation with moderate to high doses of the virus; the dose required is dependent on the LCMV isolate used (2, 5). Here too, persistent infection results from lack of viral clearance by LCMV-specific CTL. However, the basis of the CTL deficit initiated in adult animals differs from that in neonatally or in utero infected mice, as they exhibit a generalized and not just a LCMV-specific immunodeficiency and are unable to mount efficient immune responses to other viruses (54, 55, 64), parasites (68), or tumors (29).

To elucidate the mechanism(s) involved in the induction of a generalized immune suppression by LCMV in adult mice, we compared the interaction of two LCMV isolates with the immune system in vivo. One of these isolates does and the other does not induce a generalized immune suppression following i.v. inoculation of 2×10^6 PFU into adult mice. The LCMV genome is composed of two RNA segments, a short (S) seg-

* Corresponding author. Mailing address: Department of Neuropharmacology, Division of Virology (IMM6), The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-9602. Fax: (619) 554-9981.

TABLE 1. Comparison of the ability of splenocytes from mice infected with LCMV Armstrong and clone 13 to proliferate in response to stimulation with PHA[a]

Source of splenocytes and mouse number	Mean [^{3}H]thymidine incorporation (cpm)	
	No PHA	PHA (5 μg/ml)
Uninfected mice		
1	698	17,841
2	782	14,823
3	680	12,207
4	972	18,986
Armstrong-infected mice		
1	3,472	19,468
2	4,211	25,280
3	5,242	22,272
4	4,758	22,310
Clone 13-infected mice		
1	1,173	18,590
2	2,395	20,266
3	2,122	22,956

[a] Splenocytes prepared from uninfected mice and animals infected i.v. 28 days previously with LCMV Armstrong or clone 13 were cultured at 2×10^5 per well with or without 5 μg of PHA per ml for 3 days and were pulsed with [^{3}H]thymidine for the final 18 h of the culture period. Results shown are the mean [^{3}H]thymidine incorporation, expressed as counts per minute, of triplicate wells. The variance was always <10%.

ment that encodes the viral glycoprotein precursor (GP-C) (which is posttranslationally processed to yield the two virion glycoproteins, GP-1 and GP-2) and nucleoprotein (NP) genes and a long (L) segment that encodes the viral polymerase (L) and Z genes (53, 57). The virus isolates used are closely related in that the immunosuppressive LCMV isolate (clone 13) was derived from the spleen of a mouse persistently infected from birth with the nonsuppressive LCMV Armstrong 53b isolate (2), and differs from it at only two amino acid positions, amino acid 260 in the virus glycoprotein GP-1 and amino acid 1079 in the viral polymerase protein, L (1, 56, 58). Sequence analysis of a panel of independently-isolated immunosuppressive variants derived from Armstrong-infected mice has shown that only the amino acid change in GP-1 is consistently associated with the immunosuppressive phenotype (1, 56), while reassortant analysis suggests that the L RNA sequence also affects the biological properties of the virus (3, 37).

We report here that the immune suppression induced by LCMV clone 13 is associated with a CD8-dependent loss of interdigitating dendritic cells from periarteriolar lymphoid sheaths (PALS) in the spleen and, functionally, a deficit in the ability of splenocytes from infected mice to stimulate the proliferation of naive T cells in a primary mixed lymphocyte reaction. Investigating the mechanism which underlies the depletion of interdigitating dendritic cells in clone 13 but not Armstrong-infected mice, we show that LCMV Armstrong and clone 13 exhibit differences in their tropism within the spleen. Notably, clone 13 causes a higher level of infection of periarterial interdigitating dendritic cells in the white pulp than Armstrong. The consequence of this is that large-scale destruction of the clone 13-infected dendritic cells, which are targets for the antiviral CD8$^+$ CTL response, then occurs. We discuss the importance of in vivo tropism in determining virus pathogenesis and the role that dendritic cell infection may play in the immune suppression associated with clinically important human viral infections, including HIV-1.

MATERIALS AND METHODS

Virus. The parental Armstrong 53b strain of LCMV is a triple-plaque-purified clone from Armstrong CA 1371 (21), subsequently maintained by passage in baby hamster kidney (BHK) cells. Clone 13 is a triple-plaque-purified variant of this strain derived from spleen cells of an adult BALB/WEHI mouse persistently infected from birth with Armstrong 53b (2). Clone 13 stocks were also grown in BHK cells. The titers of LCMV stocks were determined by plaque assay on Vero cells (21). All virus stocks were free of mycoplasma contamination as judged by Hoechst staining of cells growing in antibiotic-free medium 48 h after virus infection.

Mice: infection and in vivo depletion of CD8$^+$ T cells. BALB/c ByJ and C57BL/6 mice maintained in the closed breeding colony of The Scripps Research Institute were used for all experiments. BALB/c ByJ mice were infected as adults (7 to 10 weeks old) by i.v. inoculation of 2×10^6 PFU of virus. In some

TABLE 2. Comparison of the ability of splenocytes from mice infected with LCMV Armstrong and clone 13 to act as antigen-presenting cells for stimulation of a primary MLR[a]

Source of splenocyte APC	Mean [^{3}H]thymidine incorporation (cpm)			
	Expt 1		Expt 2	
	APC only	APC + T cells	APC only	APC + T cells
No APC		88		208
Uninfected mice				
1	113	32,476	83	22,074
2	162	26,521	245	31,766
3	277	23,396		
4	213	23,641		
Armstrong-infected mice				
1	206	27,756	357	32,818
2	196	25,552	432	67,736
3	109	22,216	306	38,649
4	199	25,726		
Clone 13-infected mice				
1	110	1,701	161	8,850
2	130	1,119	204	5,665
3	42	724	120	9,493
4	80	3,108		

[a] Irradiated splenocytes prepared from uninfected BALB/c ByJ (*H-2^d*) mice and mice infected i.v. 28 days previously with LCMV Armstrong or clone 13 were cultured at 4.5×10^5 per well (experiment 1) or 4.0×10^5 per well (experiment 2) with and without 2×10^5 antigen-presenting cell (APC)-depleted lymph node T cells from uninfected C57BL/6 (H-2^b) mice for 5 days. Wells were pulsed with [^{3}H]thymidine for the final 18 h of the culture period. Results shown are the mean [^{3}H]thymidine incorporation (in counts per minute) of triplicate wells. The variance was always <10%.

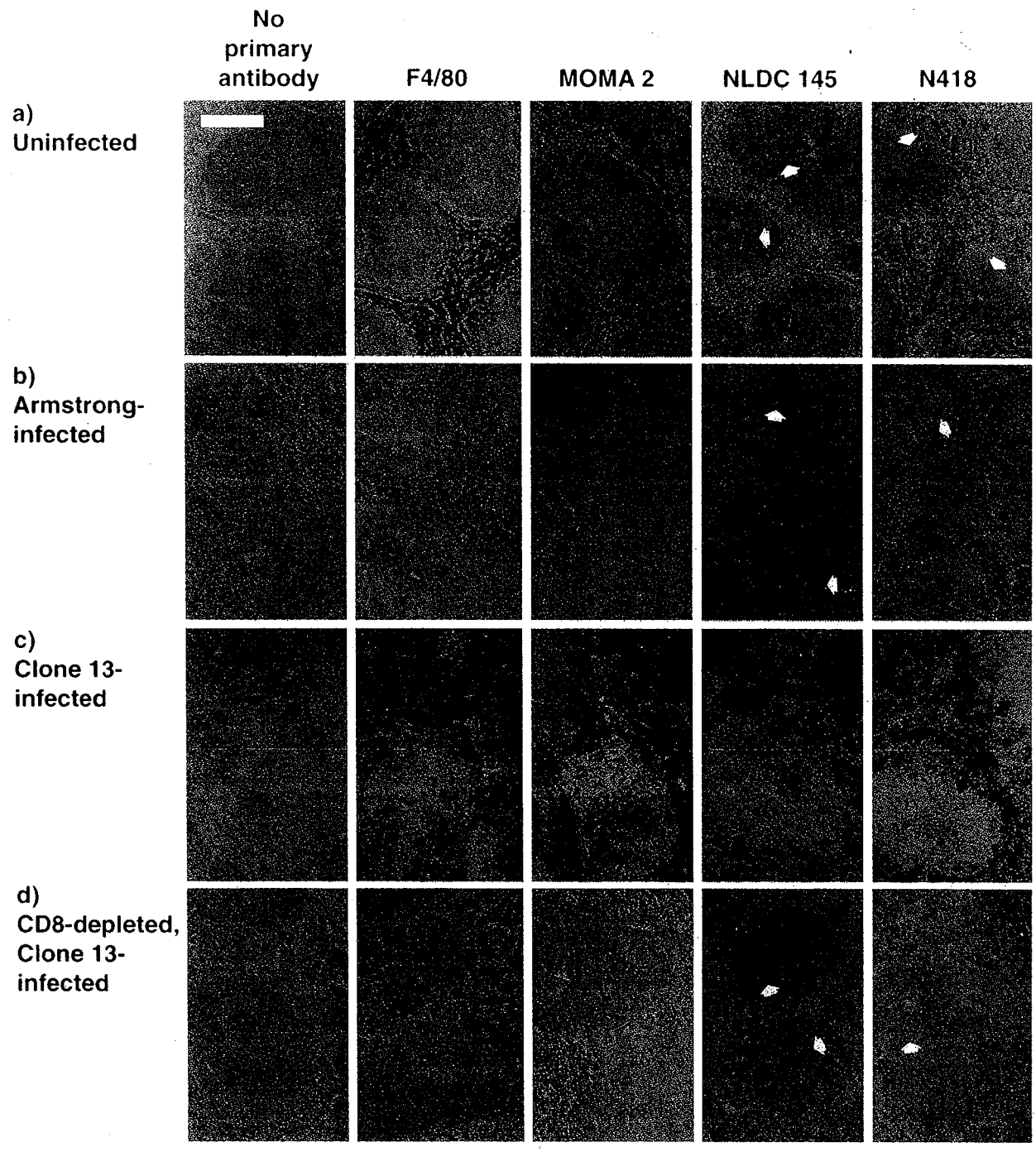

FIG. 1. Immunohistochemical staining of spleen sections from uninfected, Armstrong-infected, clone 13-infected, and CD8-depleted, clone 13-infected mice with antibodies to macrophages and dendritic cells. Spleens were cryopreserved from uninfected BALB/c ByJ mice (a), age-matched mice inoculated 7 days previously with 2×10^6 PFU of LCMV Armstrong (b) or clone 13 i.v. (c), and CD8-depleted animals similarly infected with clone 13 (d). Cryosections (10 μm) were immunostained as detailed in the Materials and Methods section with no primary antibody (as a negative control); F4/80, a monoclonal antibody to mature mouse macrophages (7); MOMA 2, a pan-macrophage marker (31); NLDC 145, which recognizes a subpopulation of murine dendritic cells, the interdigitating cells in the central periarterial sheaths (12, 30); or N418, an antibody to murine CD11c (40), which is expressed at high levels in the steady state in the spleen primarily on dendritic cells. The chromagen used in the immunostaining procedure was diaminobenzidine, which gives a brown product where primary antibody bound; sections were counterstained with Mayer's hematoxylin. Bar, 100 μm. All panels are shown at the same magnification. White arrows indicate interdigitating periarterial dendritic cells in the PALS, and black arrows indicate disintegrated white pulp with a lack of interdigitating periarterial dendritic cells in clone 13-infected mice which were not CD8-depleted. NLDC 145 staining is illustrated at higher power in other sections in Fig. 2.

experiments, mice were depleted of $CD8^+$ T cells in vivo by treatment with the anti-CD8 rat monoclonal antibody YTS 169.4 (18). The antibody was partially purified from ascites by ammonium sulfate precipitation, dialyzed against phosphate-buffered saline (PBS), and adjusted to 10 mg/ml. Mice were inoculated i.v. with 1 mg of antibody on day −1 and +3 relative to the time of virus infection (day 0). The efficiency of depletion of $CD8^+$ T cells was checked by immunofluorescent staining of peripheral blood smears on day 7 postinfection when the mice were sacrificed and was >95%.

Assays for T-cell proliferation in a mixed lymphocyte reaction. The ability of splenocytes from uninfected mice and mice infected with LCMV Armstrong or

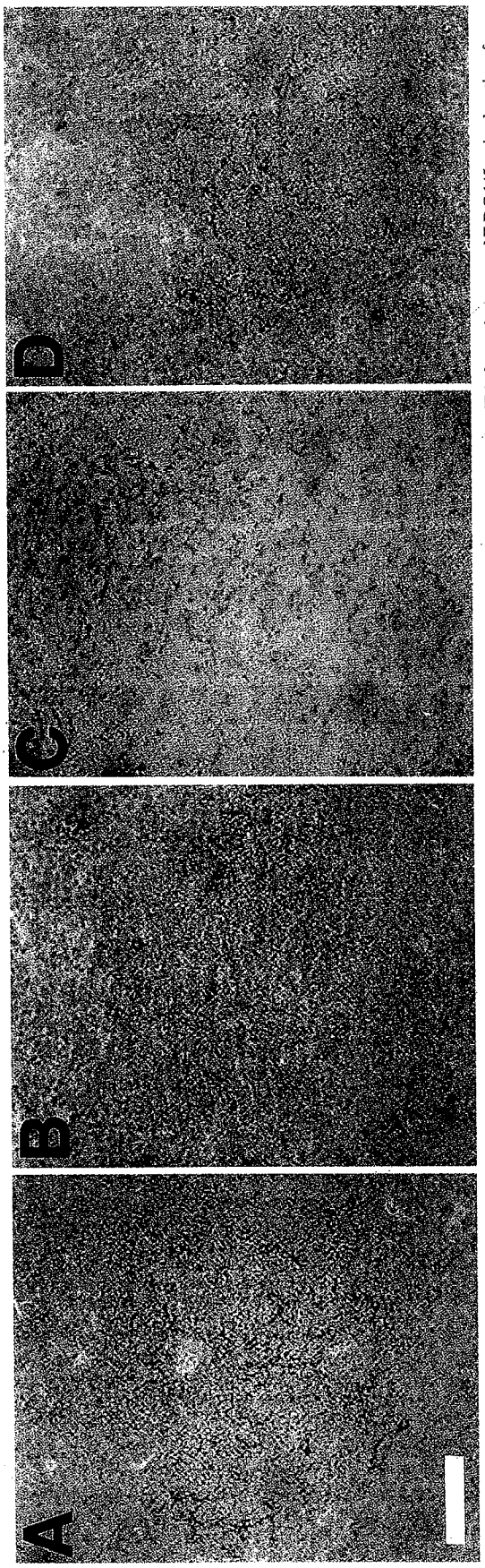

FIG. 2. Higher-power view of NLDC 145 staining to illustrate the loss of this cell subpopulation from the PALS of LCMV clone 13- but not Armstrong-infected mice. This figure shows more NLDC 145-stained sections from the experiment in Fig. 1, viewed at higher power (bar, 20 μm) to allow clearer visualization of interdigitating periarterial dendritic cells. (A) Immunohistochemical staining with NLDC 145 on a 10-μm cryosection from the spleen of an uninfected BALB/c ByJ mouse illustrates the network of interdigitating dendritic cells around the central arteriole in the T-cell area of the white pulp. (B) NLDC 145 staining of a similar section from a mouse infected 7 days previously with LCMV Armstrong reveals that although the spleen is somewhat disorganized because of the ongoing immune response, NLDC 145-positive cells are still present. (C) NLDC 145 staining of a similar section from a mouse infected 7 days previously with LCMV clone 13 shows that no NLDC 145-positive cells remain in this area of disintegrated white pulp. (D) Section from a mouse depleted of $CD8^+$ T cells prior to infection with LCMV clone 13 stained with NLDC 145 shows that the network of interdigitating periarterial dendritic cells remains intact.

clone 13 to act as accessory cells for T-cell stimulation in a one-way mixed lymphocyte reaction (MLR) was compared in [^{3}H]thymidine incorporation assays. The responding cells were lymph node T cells from uninfected C57BL/6 (H-2^b) mice purified as described by Sprent and Schaefer (60). Briefly, cell suspensions were prepared from pooled axillary, inguinal, cervical, periaortic, and mesenteric lymph nodes by mashing against wire gauze with a rubber plunger. After being washed three times in R2.5 medium (RPMI 1640 medium supplemented with 2.5% fetal bovine serum [FBS]), cells were resuspended at 2×10^7/ml in RPMI 1640 medium supplemented with 5% FBS, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), monoclonal antibodies J11d (recognizes a heat-stable antigen on B cells but not mature T cells [13]; rat immunoglobulin M [IgM]; tissue culture supernatant used at a final dilution of 1/5) and 28-16-8S (an anti-I-A^b antibody that cross-reacts with I-A^d [50]; mouse IgM; tissue culture supernatant used at a final dilution of 1/50), guinea pig complement (used at a final dilution of 1/10), and rabbit complement (used at a final dilution of 1/80). The cells were incubated for 45 min in a 37°C water bath, washed four times in R2.5, and then resuspended in proliferation assay medium consisting of RPMI 1640 supplemented with 50 U of penicillin per ml, 50 μg of streptomycin per ml, 1% glutamine, 10 mM HEPES, 10^{-5} M 2-mercaptoethanol, 10% FBS, and 5% NCTC 109 (Bio-Whittaker, Walkersville, Md.). Accessory cells were prepared from the spleens of uninfected BALB/c ByJ (H-2^d) mice and BALB/c ByJ mice infected i.v. with 2×10^6 PFU of LCMV Armstrong or clone 13 28 days previously. Splenocyte suspensions obtained by mashing spleens against wire gauze with a rubber plunger were washed three times in R2.5, γ-irradiated (2,000 rads), and resuspended in proliferation assay medium. In 96-well flat-bottom plates, accessory cells were set up at 0, 2×10^5, 4×10^5, or 8×10^5 per well, and 0, 1×10^5, 2×10^5, or 5×10^5 responder cells were added, respectively, in a total volume of 200 μl per well. All variables were assayed in triplicate. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 5 days. During the final 18 h of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine per well (6.7 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) and then harvested onto glass fiber filters using a semiautomated cell harvester (Cambridge Technology, Inc., Watertown, Mass.). The incorporated radioactivity was determined by using a liquid scintillation beta counter (Beckman Instruments, Inc., Fullerton, Calif.). Results shown are the mean [^{3}H]thymidine incorporation (mean counts per minute) of triplicate wells.

Assays for T-cell proliferation in response to PHA stimulation. The ability of splenocytes from uninfected mice and mice infected with LCMV Armstrong or clone 13 to proliferate in response to stimulation with phytohemagglutinin (PHA) was compared in [^{3}H]thymidine incorporation assays. Splenocyte suspensions prepared as described for accessory cells in the MLR but not γ-irradiated were cultured at 2×10^5 or 5×10^5 per well in 96-well round-bottom plates in a total volume of 200 μl of proliferation assay medium per well containing PHA (Sigma, St. Louis, Mo.) at 0, 2.5, 5, or 10 μg/ml for 3 days. Cells were pulsed with [^{3}H]thymidine during the final 18 h of culture and harvested, and incorporated radioactivity was determined as described above.

CTL assays. LCMV-specific CTL activity was quantitated in standard ^{51}Cr release assays as described by Byrne and Oldstone (15). Effector cells were splenocyte suspensions from mice infected 5 or 7 days previously with LCMV Armstrong or clone 13 which were depleted of erythrocytes by incubation for 5 min in 0.83% NH_4Cl, washed four times in R2.5 medium, and filtered through 45- to 60-mm-pore-size nylon mesh to remove aggregates. Target cells were ^{51}Cr-labeled fibroblast cell lines BALB Cl 7 (H-2^d) and MC57 (H-2^b) either uninfected or infected at a multiplicity of infection of 3 PFU per cell with LCMV Armstrong 48 h earlier. Target cells were plated at 10^4 per well, and effectors were added to give effector:target ratios of 50:1 and 25:1. All variables were set up in triplicate, and the assay time was 5 h. Results are expressed as the percent specific ^{51}Cr release, calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Immunohistochemical staining. Spleens removed from uninfected BALB/c ByJ mice and at different times following infection of mice with LCMV Armstrong or clone 13 were embedded in Tissue-Tek OCT compound (Miles Diagnostics Division, Elkhart, Ind.), snap frozen in isopentane-dry ice, and stored at −20°C. Immunohistochemical staining was performed on 10-μm cryostat sections by a method based on that described by Lo et al. (34). Briefly, sections were fixed for 30 s with 1% paraformaldehyde in PBS, rinsed in PBS, blocked with avidin and biotin solutions (Vector Laboratories, Inc., Burlingame, Calif.), rinsed again in PBS, and then incubated with primary antibodies or PBS only as a control for 1 h at room temperature. Primary antibodies used were F4/80, a rat IgG2b monoclonal antibody to mature mouse macrophages (7) (Serotec, Oxford, United Kingdom; tissue culture supernatant used at 1/10); MOMA 2, a rat IgG2b monoclonal antibody to a murine pan-macrophage marker (31) (Serotec; tissue culture supernatant used at 1/10); Ser 4, a rat IgG2a monoclonal antibody to murine marginal zone macrophages (20) (a gift from S. Gordon, Oxford, United Kingdom; tissue culture supernatant used at 1/10); N418, a hamster monoclonal antibody that reacts with mouse CD11c (40) (tissue culture supernatant prepared from the hybridoma HB 224, obtained from the American Type Culture Collection, Rockville, Md.; used undiluted); and NLDC 145, a rat IgG2a monoclonal antibody which recognizes a subpopulation of murine dendritic cells (12, 30) (freeze-dried antibody obtained from Accurate Chemical and Scientific Corporation, Westbury, N.Y.; reconstituted as per the manufacturer's directions and then used at 1/50). After being washed in PBS, rat and hamster antibodies were

TABLE 3. Levels of LCMV-specific, MHC-restricted CTL activity mediated by splenocytes of mice inoculated with LCMV Armstrong or clone 13[a]

Source of effector splenocytes [virus inoculated (days postinfection)]	Target cells					
	$H\text{-}2^d$				$H\text{-}2^b$	
	Uninfected		LCMV infected		LCMV infected	
	50:1	25:1	50:1	25:1	50:1	25:1
Armstrong (5)	13.9	13.3	63.7	42.5	4.8	3.3
	7.5	8.8	38.2	26.0	0.0	1.2
Clone 13 (5)	16.0	9.5	42.2	27.4	2.9	0.0
	14.0	9.7	52.4	38.6	3.3	2.3
Armstrong (7)	2.2	1.9	49.9	33.4	2.9	2.3
	0.0	0.0	52.5	35.1	0.4	0.0
Clone 13 (7)	0.0	1.3	9.3	5.4	2.3	2.0
	0.0	0.1	9.6	7.5	1.7	0.6

[a] The ability of splenocytes prepared 5 or 7 days following i.v. inoculation of BALB/c ByJ ($H\text{-}2^d$) mice with LCMV Armstrong or clone 13 to mediate MHC-restricted, virus-specific CTL lysis was tested by a ^{51}Cr release assay. Target cells were MHC-matched ($H\text{-}2^d$) or mismatched ($H\text{-}2^b$) fibroblast cell lines either uninfected or infected at a multiplicity of infection of 3 PFU per cell with LCMV Armstrong 48 h prior to the assay. As there are no amino acid differences between LCMV Armstrong and clone 13 in the CTL epitopes recognized on the $H\text{-}2^d$ or $H\text{-}2^b$ backgrounds (58), Armstrong-infected target cells are recognized equally well by CTL raised to LCMV Armstrong and clone 13. The results shown are the mean (of triplicate wells) percent specific ^{51}Cr release (calculated as described in Materials and Methods) mediated by effector splenocytes from individual animals at effector:target ratios of 50:1 and 25:1. The variance was always <10%.

followed by biotinylated $F(ab')_2$ mouse anti-rat or goat anti-hamster IgG, respectively (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.; used at 1/500), for 30 min at room temperature. Slides were then washed in PBS, incubated for a further hour at room temperature with streptavidin peroxidase (Jackson Immunoresearch Laboratories; freeze-dried antibody reconstituted as per the manufacturer's directions and then used at 1/500), and washed again in PBS, and then staining was detected with diaminobenzidine as a chromagen. Finally, sections were counterstained with Mayer's hematoxylin (Sigma) and mounted in Aqua-Mount (Lerner Laboratories, Pittsburgh, Pa.).

Combined immunohistochemical staining and in situ hybridization. Immunohistochemical staining was performed on 10-μm cryostat sections of spleens as described above. After the color reaction, sections were not counterstained but instead were dehydrated by sequential 5-min incubations in 50, 75, and 95% ethanol plus 0.33 M sodium acetate, air dried, and stored at room temperature overnight before in situ hybridization was carried out. In situ hybridization was performed with a ^{35}S-labeled riboprobe specific for the LCMV nucleoprotein (NP) gene, a gene in which there are no sequence differences between LCMV Armstrong and clone 13 (58), by the method described in Wilson and Higgins (66). Briefly, the riboprobe was prepared by transcription from the T7 promoter of the plasmid NP bluescript, a plasmid created by cloning the 1,164-bp *Bgl*II fragment from a cDNA of the LCMV Armstrong S RNA segment (59) into the plasmid bluescript KS (Stratagene, La Jolla, Calif.). Transcription from the T7 promoter of NP bluescript generates a single-stranded RNA product complementary to the LCMV NP mRNA and antigenomic sequence. Transcription was carried out using the Promega transcription in vitro system (Promega, Madison, Wis.), employing ^{35}S-CTP (800 Ci/mmol; Amersham, Arlington Heights, Ill.). The transcription product was hydrolyzed with 0.25 M NaOH, neutralized, and phenol-chloroform extracted, and unincorporated nucleotides were separated from the probe by using a nick column (Pharmacia, Alameda, Calif.). The probe was then ethanol precipitated, washed, and resuspended in water, and the percent incorporation of radiolabel was measured to allow calculation of the quantity of probe generated. Probes were stored at −70°C before use. Prior to hybridization, sections were fixed in 4% buffered formaldehyde for 5 min at room temperature, washed in PBS, deproteinated by incubation for 7.5 min at 37°C in 0.025 mg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) per ml, washed in PBS, and incubated in 0.05 N HCl for 7.5 min at room temperature. Following another wash in PBS, sections were fixed for a further 2 min at room temperature in 4% buffered formaldehyde, washed in PBS, dehydrated by sequential 2-min incubations in 60, 80, and 95% ethanol plus 0.33 M sodium acetate, and allowed to air dry. The sections were then covered with prehybridization mix {50% deionized formamide, 750 mM NaCl, 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 10 mM EDTA, 10% dextran sulfate, 5× Denhardt's solution, 50 mM dithiothreitol, 0.2% sodium dodecyl sulfate, plus 0.1 mg of salmon sperm DNA (Sigma) per ml and 0.1 mg of yeast tRNA (BRL, Gaithersburg, Md.) per ml}, and incubated for approximately 3 h at 50°C in a moist box. The prehybridization mix was then decanted, hybridization mix was added (prehybridization mix containing 0.05 μg of the ^{35}S-labeled riboprobe per ml), and the slides were covered with coverslips and incubated overnight (12 to 16 h) at 50°C in a moistbox. The coverslips were removed, and the slides were washed in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 70 mM 2-mercaptoethanol for 15 min at room temperature and then for 15 min in 4× SSC only. A 15-min incubation at 37°C in 20 μg of pancreatic RNase (Calbiochem, La Jolla, Calif.) per ml in 0.5 M NaCl–10 mM Tris (pH 7.5)–1 mM EDTA was followed by a wash in 2× SSC for 15 min at 56°C; then slides were dehydrated as described above and air dried. Slides were then dipped in photographic emulsion (Eastman Kodak Co., Rochester, N.Y.) and developed 2 weeks later. Finally, they were counterstained in Mayer's hematoxylin (Sigma), dehydrated to 70% ethanol, counterstained in eosin (Sigma), washed in 70, 95, and 100% ethanol, cleared in xylene, and mounted in Flo-texx mountant (Lerner Laboratories).

Isolation of splenic dendritic cell subpopulations and determination of the proportion of cells infected by LCMV. Cell suspensions were prepared from the spleens of mice infected 3 days previously with LCMV Armstrong or clone 13 by collagenase digestion following the method of Swiggard et al. (62). As these authors describe, this method increases the yield of dendritic cells two- to threefold over disruption of spleens by nonenzymatic means and also helps to release dendritic cells that lie within periarterial sheaths. In some experiments, dendritic cells were enriched from the collagenase-digested splenocyte suspension by plastic adherence (62); in others, they were isolated directly from the total splenocyte population so that any nonadherent dendritic cells would not be excluded. Different dendritic cell subpopulations were then isolated by fluorescence-activated cell sorting (FACS) (11). Briefly, cells were immunostained by using as primary antibodies 33D1, a rat IgG2b monoclonal antibody against murine lymphoid dendritic cells (44) (ascites prepared by standard techniques using the hybridoma TIB 227 obtained from the American Type Culture Collection; used at 1/50), or NLDC 145 (tissue culture supernatant used at 1/10), described above; and as a second antibody fluorescein isothiocyanate-labeled $F(ab')_2$ mouse anti-rat IgG (Jackson Immunoresearch Laboratories). Cell populations were also labeled with propidium iodide to allow the exclusion of dead cells from the analysis. Flow cytometry was performed on a FACS IV (Becton Dickinson & Co., Mountain View, Calif.). Between 5×10^4 and 1×10^5 positive cells were collected from each sample. The purity of each subset always exceeded 95%. Sorted cells were pelleted and resuspended in PBS at approximately 10^6/ml, spotted onto Fisher Plus slides (Fisher Scientific, Pittsburgh, Pa.), and allowed to air dry. Slides were fixed in 4% buffered paraformaldehyde for 5 to 7 min, air dried, and stored at room temperature. LCMV-infected cells were identified by in situ hybridization using a ^{35}S-labeled riboprobe specific for LCMV NP mRNA, as described above. The percentage of infected cells was determined by viewing the slides at 200X magnification with an Olympus BH-2 microscope and counting in at least six fields of >100 cells each the proportion of cells with >10 grains of in situ signal above them (the background level of signal was <5 grains per cell, as determined by using uninfected cell populations).

RESULTS

LCMV clone 13-infected mice exhibiting a generalized immune suppression have a deficit in splenic antigen-presenting cells capable of stimulating a primary MLR. To investigate the basis of the generalized immune suppression induced in adult mice by LCMV clone 13, the ability of splenocytes from mice infected when 8 weeks old with the immunosuppressive LCMV clone 13 isolate or the nonsuppressive parental strain, LCMV Armstrong, to act as accessory or antigen-presenting cells was

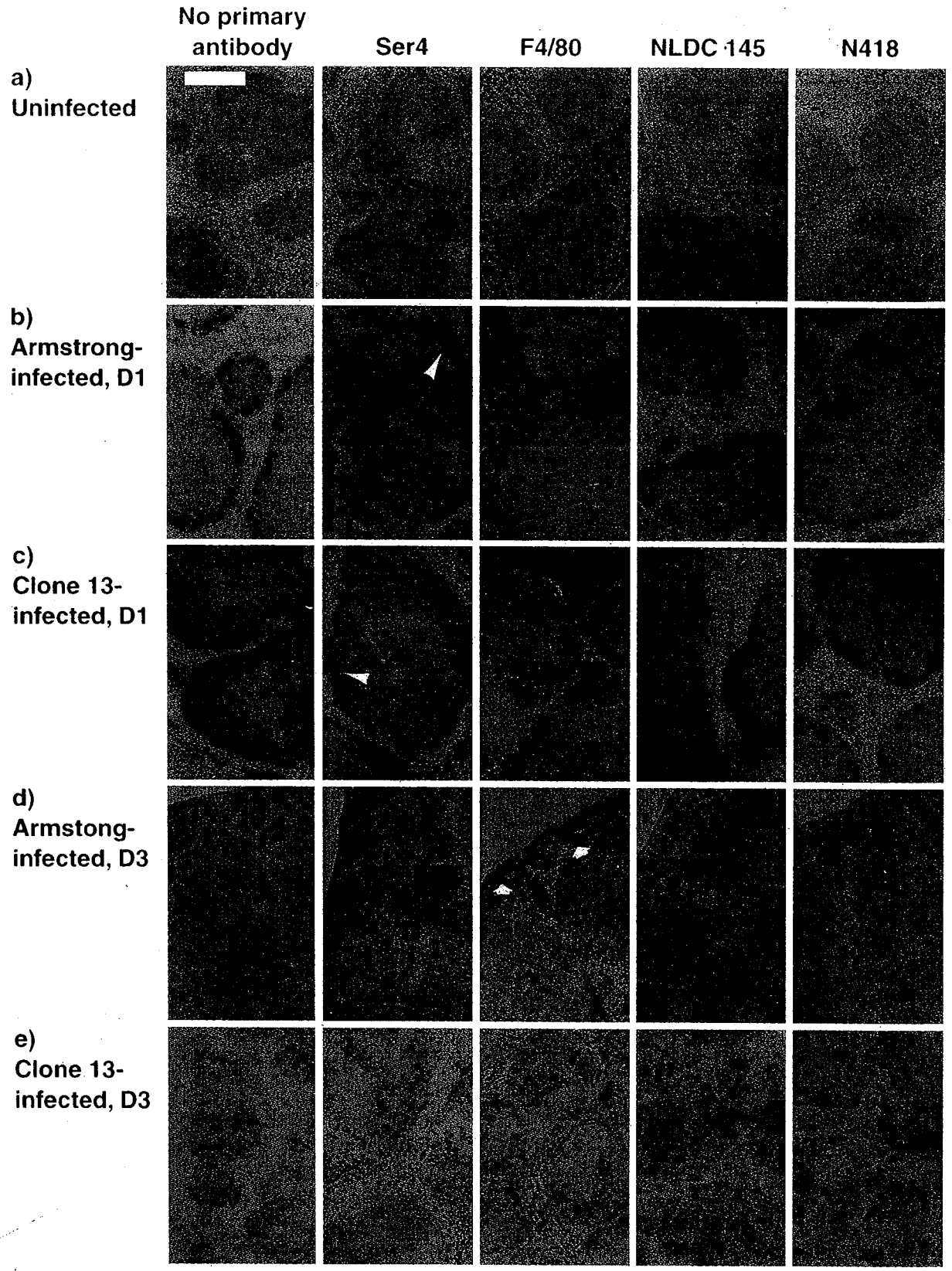

FIG. 3. Combined immunohistochemical staining to identify splenocyte subpopulations and in situ hybridization to locate virus-infected cells on spleen sections from uninfected mice and mice infected 1 or 3 days previously with LCMV Armstrong or clone 13. Spleens were cryopreserved from uninfected BALB/c ByJ mice and age-matched mice infected i.v. with 2×10^6 PFU of LCMV Armstrong or clone 13, 1 or 3 days previously. Cryosections (10 μm) were first immunostained with no primary antibody (as a negative control); Ser 4, a monoclonal antibody which recognizes murine marginal zone macrophages (20); or F4/80, NLDC 145 or N418 (see legend to Fig. 1). In situ hybridization was then carried out using a ^{35}S-labeled riboprobe specific for LCMV NP mRNA, in which there are no sequence differences

compared in two in vitro assays. First, the ability of splenocytes from mice infected 28 days previously with LCMV Armstrong or clone 13 to proliferate in response to stimulation with PHA was tested. As shown in Table 1, the PHA-induced T-cell proliferation response of splenocytes from clone 13-infected mice was comparable to that of splenocytes from Armstrong-infected mice or uninfected animals. This indicated that in immunosuppressed animals T-cell responsiveness to mitogen stimulation was intact and that there was sufficient accessory cell function to support this response. Next, the ability of irradiated splenocytes from uninfected BALB/c ByJ ($H\text{-}2^d$) mice and mice infected 28 days previously with LCMV Armstrong or clone 13 to induce the proliferation of accessory cell-depleted $H\text{-}2^b$ T cells in a primary MLR was assessed. As the data in Table 2 from two representative experiments illustrate, splenocytes from clone 13-infected mice were significantly less efficient at stimulating a primary MLR than splenocytes from uninfected or Armstrong-infected mice. This was not due to the presence of persisting virus in the splenocyte preparations from clone 13-infected mice, as splenocytes from mice persistently infected with LCMV clone 13 from birth exhibit no defect in antigen-presenting cell function despite the presence of persisting virus (reference 64 and data not shown). Rather, the data demonstrate that there is a functional deficit in antigen-presenting cells capable of stimulating a primary MLR in clone 13-infected mice.

The functional deficit in antigen-presenting cell activity in immunosuppressed mice is associated with a loss of interdigitating dendritic cells from lymphoid follicles. Of the many antigen-presenting cell subpopulations in the spleen, only dendritic cells are capable of efficiently presenting antigen to naive T cells (reviewed in reference 61). The basis of the observed functional deficit in the ability of splenocytes from clone 13-infected mice to stimulate a primary MLR was thus likely either a loss of splenic dendritic cells or an impairment of their functional capacity. To determine whether there was a depletion of splenic dendritic cells from mice infected with LCMV clone 13, immunohistochemical staining was performed by using macrophage- and dendritic cell-specific antibodies. Spleens were removed at day 7 postinfection, when immunosuppression is first observed. Sections from uninfected mice and more than 10 individual animals infected with LCMV Armstrong or clone 13 were examined. Representative results are shown in Fig. 1a to c and 2. Staining with MOMA 2, a pan-macrophage marker (31), and F4/80, an antibody which recognizes mature macrophages (7), revealed that there was not a significant depletion of cells expressing these markers from mice infected with the immunosuppressive virus. The level of staining with MOMA 2 in the spleens of mice infected with either LCMV Armstrong or clone 13 was similar to that in uninfected mice. As F4/80 expression is known to be downregulated by inflammatory stimuli and by exposure to lymphokines, especially gamma interferon (23), it was not surprising that the intensity of F4/80 staining in the spleens of LCMV-infected mice was reduced compared with that in uninfected controls. However, the overall number of F4/80-positive cells in the spleens of mice infected with LCMV clone 13 was similar to that observed in the spleens of mice infected with the nonsuppressive parental Armstrong virus. By contrast, staining with NLDC 145, an antibody which recognizes a subpopulation of long-lived splenic dendritic cells (the interdigitating cells in the central periarterial sheaths [12, 30]), revealed a substantial loss of these cells from spleens of mice infected with clone 13 (Fig. 1c and higher-power pictures in Fig. 2). Although mice infected with LCMV Armstrong are undergoing an immune response with associated activation-induced changes in the spleen, NLDC 145-positive cells were clearly present within the white pulp (Fig. 2B). In clone 13-infected mice, however, NLDC 145-positive cells were completely lost from much of the white pulp, and the PALS in these areas appeared morphologically to have disintegrated (Fig. 2C). As the organization of both the T- and B-cell areas of the PALS was destroyed, likely not only NLDC 145-positive periarterial interdigitating dendritic cells in the T-cell areas but also follicular dendritic cells in the B-cell areas were depleted in clone 13-infected mice. Figure 1c illustrates that NLDC 145-positive cell depletion was not absolute. These cells remained and the architecture was retained in 10 to 50% of the PALS areas in the sections examined. Loss of periarterial interdigitating dendritic cells from the white pulp in the spleens of clone 13-infected mice was confirmed by staining with N418, an antibody to murine CD11c. In the steady state in the spleen, the main cells expressing high levels of CD11c are dendritic cells—both the NLDC 145-positive subpopulation of interdigitating cells in the central periarterial sheaths and the more short-lived population of dendritic cells which lie in the periphery of the T-cell area interrupting the marginal zone of macrophages (61). Both periarterial and peripheral N418-positive cells were present in Armstrong-infected mice, whereas in clone 13-infected mice there was a loss of periarterial N418 staining (Fig. 1). The N418-positive cells in the marginal zone and red pulp of clone 13-infected mice may be peripheral dendritic cells or other cell types such as macrophages on which CD11c can be upregulated during an immune response.

Loss of interdigitating periarterial dendritic cells from clone 13-infected mice is CD8-dependent and clone 13-infected mice mount a short-lived antiviral CTL response at early times postinfection. Further experiments investigated the mechanism by which interdigitating periarterial dendritic cells are depleted following infection of mice with LCMV clone 13. First, it was shown that dendritic cell loss was CD8 dependent, as in vivo depletion of $CD8^+$ T cells using a subset-specific monoclonal antibody prior to infection of mice with LCMV clone 13 prevented the NLDC 145-positive cell loss and associated disintegration of the PALS which occurred in undepleted mice (Fig. 1d and 2D). As the LCMV-specific CTL response is mediated by $CD8^+$ T cells (reviewed in reference 46), this finding raised the question of whether the $CD8^+$-dependent immunopathological damage might be mediated by virus-specific CTL. The immunosuppressive clone 13 LCMV variant was initially characterized as a virus which is able to establish a persistent infection in adult immunocompetent mice because the CTL response generated to it is insufficient to achieve viral clearance (2). Indeed, 7 days postinfection when the CTL response to LCMV Armstrong peaks, only low levels of LCMV-specific CTL activity are detected in clone 13-in-

between Armstrong and clone 13 (58). Finally, sections were counterstained with hematoxylin and eosin. Bar, 100 μm. White arrowheads indicate colocalization of in situ signal with Ser 4 staining in the spleens of mice infected 1 day previously with LCMV Armstrong or clone 13. Small white arrows indicate in situ signal colocalizing with F4/80 staining in the red pulp of the spleens of mice infected 3 days previously with LCMV Armstrong; and small black arrows indicate in situ signal colocalizing with periarterial interdigitating dendritic cells in the white pulp of the spleen of mice infected 3 days previously with LCMV clone 13. The different locations of viral nucleic acids in the spleens of mice infected 3 days previously with LCMV Armstrong and clone 13 are illustrated more extensively in Fig. 4, and the cell types predominantly infected by LCMV Armstrong and clone 13 on day 3 postinoculation are illustrated at higher power in other sections in Fig. 5.

FIG. 4. In situ hybridization to compare the location of viral nucleic acids within the spleen 3 days after infection of mice with LCMV Armstrong or clone 13. In situ hybridization was performed (without immunohistochemistry) on spleens from additional mice from the experiment in Fig. 3, again using a ^{35}S-labeled riboprobe specific for LCMV NP mRNA. Sections were counterstained with hematoxylin and eosin. Bar, 100 μm. (A) Spleen section from an uninfected control BALB/c ByJ mouse—no viral nucleic acids are seen. (B) Spleen section from a mouse infected i.v. with 2×10^6 PFU of LCMV Armstrong 3 days previously—viral nucleic acids are predominantly found in the red pulp. (C) Spleen section from a mouse infected i.v. with 2×10^6 PFU of LCMV clone 13 3 days previously—viral nucleic acids are predominantly found in the white pulp.

fected mice (reference 2 and Table 3). However, when the LCMV-specific CTL response mounted at earlier time points was measured, clone 13-infected mice were found to make a strong antiviral CTL response (Table 3, day 5 data). We thus hypothesized that interdigitating dendritic cells are depleted from the PALS in the spleen of clone 13-infected mice because of lysis mediated by antiviral $CD8^+$ CTL activated at early times postinfection. Since Armstrong-infected mice also mount an antiviral CTL response at early times postinfection (Table 3), this hypothesis would predict that as NLDC 145-positive cells show marked depletion from clone 13-infected mice but minimal depletion from Armstrong-infected mice (Fig. 1 and 2), clone 13 must infect a larger number of these cells than Armstrong. To test this prediction, the tropism of LCMV Armstrong and clone 13 within the spleen at early times postinfection was compared.

LCMV Armstrong and clone 13 have a differential tropism within the spleen. Combined immunohistochemical staining to identify splenocyte subpopulations and in situ hybridization to locate virus-infected cells was performed on sections of spleens cryopreserved from mice 1 and 3 days following infection with LCMV Armstrong or clone 13. Sections from more than 10 mice infected with each virus were examined at both time points, and representative results are displayed in Fig. 3 to 5. The initial patterns of infection within the spleen following i.v. inoculation of mice with 2×10^6 PFU of either LCMV Armstrong or clone 13 were similar. One day postinfection, both viruses almost exclusively infected cells in the marginal zone of the white pulp, with the in situ signal colocalizing with the immunohistochemical-staining pattern of monoclonal antibody Ser 4, a marker for marginal zone macrophages (20) (Fig. 3b and c). However, by 3 days postinoculation, although there was still some evidence of infection of Ser 4-positive cells by both viruses, Armstrong and clone 13 were predominantly localized to different areas of the spleen (Fig. 3d and e and 4). In Armstrong-infected mice, infection was heaviest at the edges of the spleen and viral nucleic acids were mainly (although not exclusively) found within the red pulp (Fig. 4B). The in situ signal largely colocalized with the F4/80 staining pattern, an antibody which identifies mature macrophages found predominantly within the red pulp (7) (Fig. 3d and 5a), although some of the infected cells in the red pulp may have been Ser 4-positive cells that had migrated out from the marginal zone. By contrast, in clone 13-infected mice the majority of the infected cells within the spleen at day 3 were found within the white pulp (Fig. 4C). The in situ signal in the T-cell areas colocalized with NLDC 145 and the periarterial N418 staining (Fig. 3e, 5B, and 5C). In addition, there were other infected cells in the B-cell areas (likely follicular dendritic cells), and there was still some infection of Ser 4-positive macrophages (Fig. 3e).

To provide further evidence that at 3 days postinoculation clone 13 causes a much higher level of infection of dendritic cells in the spleen than its nonsuppressive parental virus LCMV Armstrong, dendritic cells were isolated from splenocyte suspensions prepared from mice 3 days after infection with LCMV Armstrong or clone 13, and the proportion of cells containing viral nucleic acids was compared by in situ hybridization. Dendritic cell isolation was achieved by differential plastic adherence followed by FACS sorting after immunofluorescent staining with dendritic cell-specific monoclonal antibodies 33D1 or NLDC 145. Monoclonal antibody 33D1 was not used in the experiments in Fig. 1 and 3 because it does not work well in immunohistochemical staining. In four separate experiments (Table 4) the percentage of dendritic cells from clone 13-infected mice which were positive for viral nucleic acids was consistently higher (range, 7 to 33 times greater) than

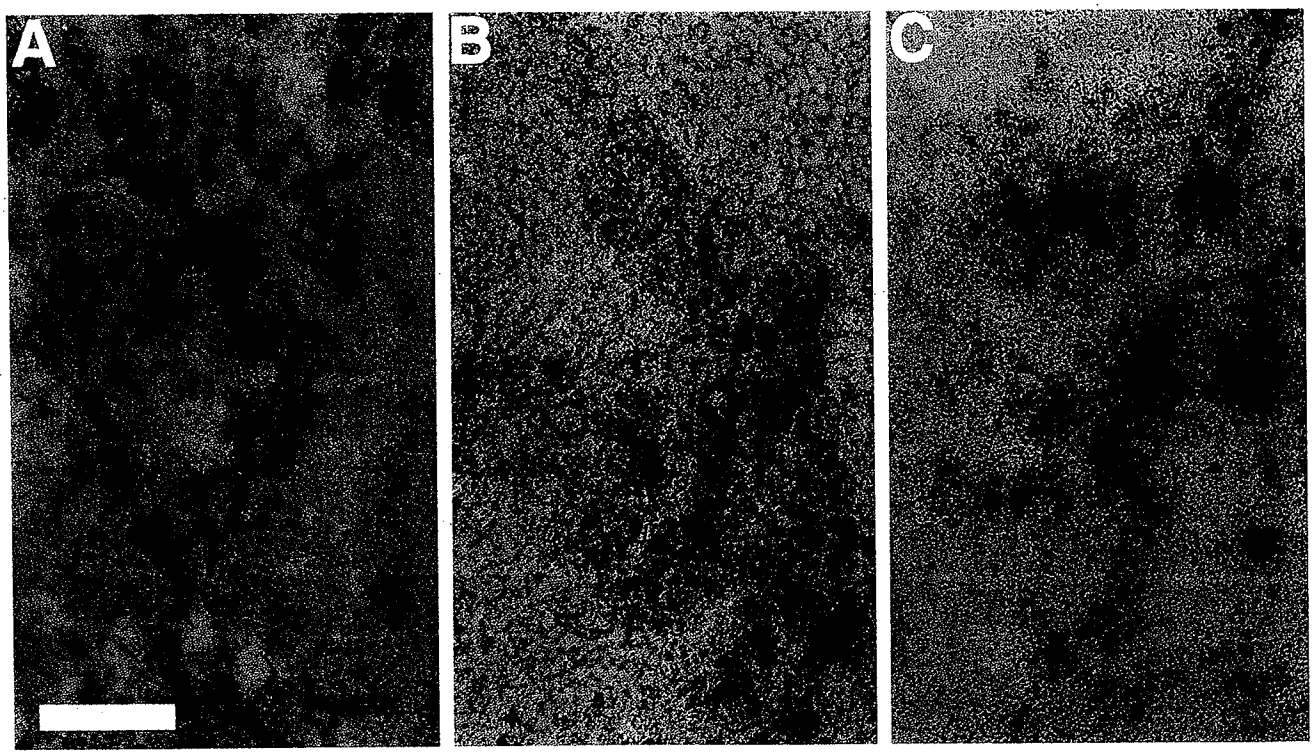

FIG. 5. Higher-power view of combined immunohistochemical staining and in situ hybridization to illustrate the cell types predominantly infected by LCMV Armstrong and clone 13 on day 3 postinoculation. This figure shows more sections from parts d and e of the experiment in Fig. 3, viewed at higher power (bar, 20 μm) to allow precise visualization of the cells with which the in situ signal is associated. (A) Immunohistochemical staining with F4/80 and in situ hybridization for LCMV NP mRNA on a 10 μm section from the spleen of a mouse infected 3 days previously with Armstrong. The in situ signal is seen to colocalize with F4/80-positive macrophages in the red pulp. (B and C) Immunohistochemical staining with NLDC 145 (B) or N418 (C) and in situ hybridization for LCMV NP mRNA on 10-μm sections from the spleen of a mouse infected 3 days previously with clone 13. The in situ signal is seen to colocalize with NLDC 145-positive interdigitating dendritic cells in the PALS (B) and periarterial N418-positive cells (C).

that from Armstrong-infected mice. It should be noted that this series of experiments was not designed to quantitate the total number of dendritic cells infected by each virus throughout the course of time. Rather, it quantitated only cells which were at a particular point in the ongoing process of spread of virus infection and simultaneous virus clearance by (nonspecific) immune defenses: namely, cells which contained replicating virus but had not started to undergo destruction (dead or damaged cells were excluded during the FACS sorting). It was thus not surprising that only a low proportion of cells scored as positive in these experiments (Table 4). The variation seen from experiment to experiment in the percentage of dendritic cells from clone 13-infected mice positive for viral nucleic acids likely reflects differences in the efficiency with which interdigitating dendritic cells were released during the collagenase digestion procedure. Dendritic cells that lie within the periarterial sheaths, the cells believed to be most extensively infected by LCMV clone 13, are difficult to release from the spleen (62). However, the results in Table 4 clearly demonstrate that clone 13 causes a higher level of infection of splenic dendritic cells than Armstrong on day 3 postinoculation, confirming the conclusions drawn from Fig. 3 to 5.

DISCUSSION

We have demonstrated that infection with a virus that has a tropism for interdigitating periarterial dendritic cells in lymphoid tissues can result in a generalized suppression of the host immune response. The mechanism by which this occurred was by virus infection, which is itself noncytolytic, rendering these critical accessory cells targets for destruction by the antiviral immune response. Our observation illustrates an important concept which is likely to apply to the immune suppression associated with other persistent virus infections, including examples of clinical importance in humans. In addition, our results demonstrate the key role that virus tropism plays in pathogenesis, as two genetically very closely related virus isolates which have differential tropisms within the spleen have widely disparate biological properties.

That the defect underlying the generalized immune suppression in clone 13-infected mice maps primarily to antigen presentation rather than T- or B-cell function per se was suggested by previous studies (5, 45) and would be predicted from the observation that clone 13 infects lymphocytes inefficiently both in vivo and in vitro (11, 37). Here we demonstrated that whereas the activity of accessory cells capable of supporting PHA-induced T-cell proliferation was not significantly impaired in clone 13-infected mice, there was a functional deficit in antigen-presenting cells capable of stimulating the proliferation of naive T cells in a primary MLR. It is well established that whereas for T-cell stimulation by many mitogens (e.g., PHA) the accessory function of antigen-presenting cells such as macrophages is comparable to that of dendritic cells, dendritic cells are remarkably more efficient than other accessory cell subpopulations in presentation of antigen to naive T cells in the initiation of a primary immune response (25, 27, 32; reviewed in reference 61). Thus, the data in Tables 1 and 2 support the hypothesis that a deficit in dendritic cell function is the basis of the immune dysfunction in clone 13-infected mice.

Further evidence for this was provided by immunohistochemical analysis. This analysis revealed that while staining with macrophage-specific markers was comparable in Armstrong and clone 13-infected mice, there was a loss of NLDC 145-positive interdigitating periarterial dendritic cells associated with disintegration of the PALS apparent only in clone 13-infected animals. Although evidence is emerging to suggest that there are distinct subsets of dendritic cells within the spleen (reviewed in reference 61), differential functions have not yet been assigned to these subsets. However, the location of NLDC 145-positive dendritic cells in the periarterial T-dependent areas of splenic white pulp is suggestive of their having an important role in the induction of T-cell-mediated immune responses. Our data lend support to this hypothesis. As illustrated in Fig. 1, NLDC 145-positive cells were not completely depleted from all regions of white pulp in clone 13-infected mice. In sections from more than 10 mice infected in several independent experiments, 10 to 50% of PALS areas were observed to still contain some NLDC 145-positive cells and retain a fairly normal architecture. This correlates with the MLR stimulation data in Table 2 showing that there is not an absolute loss of accessory cells capable of stimulating a primary T-cell response from clone 13-infected mice and that some variation is observed from animal to animal in the severity of the immune defect.

Dendritic cells were lost from clone 13-infected mice by a $CD8^+$-dependent mechanism, immunopathological destruction by the antiviral CTL response. This is in keeping with previous reports that the immune suppression induced by other LCMV isolates is caused by the $CD8^+$ antiviral CTL response (33, 42). A $CD8^+$-mediated CTL response is induced following infection of mice with both LCMV Armstrong and clone 13. Here we illustrate the key role of the differential tropism of these viruses within the spleen in determining whether or not this response has an immunopathological outcome. Both viruses initially infected cells in the marginal zone (Fig. 3). This is also the predominant location of viral antigen in the spleen of hamsters following infection with another arenavirus, Pichinde (43), suggesting that these are the first or early arenavirus-susceptible cells contacted by the virus as it spreads into the spleen from the blood. Virus infection is a dynamic process: following primary virus replication, the infection then spreads to secondary sites; and simultaneously virus may be cleared from the initial sites of infection. It is thus not surprising that by 3 days postinoculation, both Armstrong and clone 13 nucleic acids were no longer found predominantly in the same sites as on the first day postinoculation. Whereas the primary site of infection for both LCMV Armstrong and clone 13 was Ser 4-positive macrophages in the marginal zone, the cells subsequently infected by the two viruses differed. Armstrong predominantly infected red pulp macrophages, particularly at the edges of the spleen. In contrast, in clone 13-infected mice the majority of the virus was found within the white pulp (Fig. 4). Antigen-presenting cells in both B-cell areas (likely macrophages and follicular dendritic cells) and T-cell areas were infected; in the latter areas the majority of the infected cells were NLDC 145-positive interdigitating periarterial dendritic cells (Fig. 3 and 5). That clone 13 causes a much higher level of infection of splenic dendritic cells than Armstrong was confirmed by FACS sorting and in situ hybridization (Table 4). This experiment quantitated the proportion of undamaged dendritic cells which at a given time point contained viral nucleic acids; although this number was found to be low, the total number of NLDC 145-positive cells which became infected and underwent immunopathological destruction over the first 7 days postinoculation was high, as many areas of the PALS had been completely depleted of these cells by day 7 postinfection (Fig. 1 and 2). The MLR data in Table 2 also reflect the cumulative loss of dendritic cells which occurred over time as clone 13 replication was controlled. Not only interdigitating dendritic cells but also other virus-infected cells in the spleen are targets for destruction by the LCMV-specific CTL response. As the macrophages infected by Armstrong do not have the critical, irreplaceable role in the induction of immune responses that dendritic cells possess, immune-mediated clearance of Armstrong, as expected, did not result in the generalized immune suppression seen following loss of the dendritic cells infected by clone 13. Similarly, we and others have previously documented that both LCMV Armstrong and clone 13 cause a low level of infection of lymphocytes (11, 37), and that clearance of infected lymphocytes can be mediated by virus-specific CTL (11). The antiviral CTL response thus likely depleted a small number of lymphocytes from LCMV-infected mice; however, as these cells are infected to a similar extent following i.v. inoculation of mice with LCMV Armstrong and clone 13 and in both cases the number of lymphocytes infected is low, this lymphocyte loss likely does not play a major role in the generalized immune suppression seen in clone 13-infected animals. Rather, loss of white pulp antigen-presenting cells, for which clone 13 shows a preferential tropism compared with Armstrong and of which it infects a high proportion, is of key importance.

The difference in tropism between LCMV Armstrong and clone 13 was not absolute: Armstrong did cause a low level of infection of dendritic cells (Table 4). Further, we have found that LCMV Armstrong will induce a generalized immune suppression when inoculated i.v. into BALB/c mice at a very high dose (2×10^9 PFU) (8a). This would be predicted according to our hypothesis to explain the observed difference in the tropism of LCMV Armstrong and clone 13 within the spleen. Clone 13 differs from its parent virus Armstrong at only two amino acid positions, one in the virus glycoprotein GP-1 and the other in the putative viral polymerase L (56, 58). While the polymerase change contributes to the immunosuppressive phenotype (3, 37), likely by giving clone 13 a replicative advantage over Armstrong, only the phenylalanine (Armstrong) to leucine (clone 13) change in GP-1 is consistently associated with the immunosuppressive phenotype (1, 56). There is strong evidence indicating that GP-1 is the virion attachment protein that mediates binding to cell surface receptors for LCMV (9, 51, 67). Data we have obtained recently (8b) show that LCMV Armstrong and clone 13 differ in their interaction with the 120- to 140-kDa membrane protein(s) identified as a putative receptor for LCMV clone 13 (9) and suggest that LCMV Armstrong has a lower receptor binding affinity than clone 13. While this does not result in Armstrong mediating infection of fibroblast cell lines, which express high levels of the 120- to 140-kDa putative receptor protein, less efficiently than clone 13, it would confer on clone 13 a preferential ability to infect cell types that express limiting amounts of the putative receptor protein or, alternatively, are infected via a different receptor. We are currently testing the hypothesis that antigen-presenting cells, including interdigitating periarterial dendritic cells in the white pulp in the spleen, are examples of such cell types with a low receptor density of which Armstrong infects only a small proportion (unless present at extremely high doses) but clone 13 is able to infect much more efficiently.

Can our finding that virus infection of dendritic cells is a critical step in the production of immune suppression by LCMV clone 13 be generalized to other virus infections? It is of interest that all viruses known to be able to persist in vivo have been shown to infect cells of the immune system (re-

TABLE 4. Comparison of the level of infection of splenic dendritic cells by LCMV Armstrong and clone 13[a]

Expt no.	Splenocyte subset	% Infected cells[b] from mice infected with:		Difference (fold)[c]
		Armstrong	Clone 13	
1	33D1	0.2	6.7	33.5
2	33D1	<0.1	2.5	>25
3	33D1	<0.1	1.8	>18
	NLDC 145	0.15	1.4	9.3
4	33D1	1.1	11.7	10.6
	NLDC 145	1.1	7.3	6.6

[a] In each experiment, pairs of adult BALB/c ByJ mice were injected i.v. with 2×10^6 PFU of LCMV Armstrong or clone 13, and 3 days postinfection a pooled splenocyte suspension was prepared from each pair of mice by collagenase digestion (62). Dendritic cells were then isolated by differential plastic adherence and FACS sorting following immunofluorescent staining with dendritic cell-specific monoclonal antibodies 33D1 or NLDC 145. The purity of each subset always exceeded 95%. LCMV-infected cells were then identified by in situ hybridization using a ^{35}S-labeled riboprobe specific for LCMV NP, a gene in which there are no sequence differences between Armstrong and clone 13 (58).

[b] The results shown are the percentage of infected cells in each population determined by counting the number of cells with positive in situ signal in at least six fields of >100 cells each.

[c] The fold difference in level of infection of splenic dendritic cells by each virus was calculated by dividing the percentage of cells infected by clone 13 by the percentage of cells infected by Armstrong.

viewed in reference 48). Whether the immunocytes infected include dendritic cells has not thoroughly been investigated. However, this is an area of increasing interest that we and others are turning our attention to. One clinically important immunosuppressive virus infection in which dendritic cell infection has begun to be examined is that with HIV-1. Investigations on HIV-infected individuals have shown that their peripheral dendritic cells are both infected and functionally impaired (35, 36). Interestingly, in these reports, dendritic cells lose the ability to present soluble antigen and to induce allostimulation from early in asymptomatic HIV infection, whereas monocytes lose the ability to present antigen and cooperate in PHA stimulation only around the onset of symptomatic disease (17, 35, 36). While the immune system suppression associated with HIV-1 infection in humans is likely complex and multifactorial, dendritic cell infection may play an important role. Two mechanisms by which dendritic cell infection may contribute to HIV-1-induced immunosuppression are (i) by infected dendritic cells serving as a reservoir of virus in lymph nodes (6, 63) from which $CD4^+$ T lymphocytes may continually become infected and (ii) by loss or dysfunction of dendritic cells impairing the recruitment of T-helper cells into the memory pool and thus contributing to the progressive decline in $CD4^+$ lymphocytes (discussed in reference 24).

As virus-dendritic cell interactions are critically examined in other systems, the role that infection of this accessory cell subpopulation plays in virus-induced immune suppression will be clarified. In view of the central location of these cells within the immune system and their unique, critical functions in the initiation of immune responses, it is likely that virus infection of dendritic cells and subsequent impairment of their functions will prove to be an underlying factor in many examples of generalized immune suppression associated with virus infection.

ACKNOWLEDGMENTS

We thank Simon Gordon for the gift of monoclonal antibody Ser 4; David Tough for the gift of monoclonal antibodies J11d and 28-16-8S plus the complement used with them and for his critical appraisal of the manuscript; Bill Swiggard for helpful discussions about dendritic cell subpopulations and isolation; David Lo for advice on immunohistochemical staining; Ian Lipkin for advice on in situ hybridization; Victor Vedovato and Jennifer Cornell for technical assistance; Leo Fernandez, John Joubran, and Tom Knapp for FACS sorting; and Jody Anderson for typing the manuscript.

This work was supported by NIH grants AI09484 and NS12428, by AIDS Center Grant MH 47680, by Advanced Postdoctoral Fellowship FA 997-8-2 from the National Multiple Sclerosis Society (to Persephone Borrow), and by NIH Training Grant AG 00080 and National Multiple Sclerosis Society Advanced Postdoctoral Fellowship FA 1041-A-1 (to Claire Evans).

REFERENCES

1. **Ahmed, R., C. S. Hahn, T. Somasundaram, L. Villarete, M. Matloubian, and J. H. Strauss.** 1991. Molecular basis of organ-specific selection of viral variants during chronic infection. J. Virol. **65:**4242–4247.
2. **Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone.** 1984. Selection of genetic variants of LCMV in spleens of persistently infected mice. J. Exp. Med. **160:**521–540.
3. **Ahmed, R., R. S. Simon, M. Matloubian, S. R. Kolhekar, P. J. Southern, and D. M. Freedman.** 1988. Genetic analysis of in vivo-selected viral variants causing chronic infection: importance of mutation in the L RNA segment of lymphocytic choriomeningitis virus. J. Virol. **62:**3301–3308.
4. **Ahmed, R., and J. G. Stevens.** 1990. Viral persistence, p. 241–265. *In* Virology, 2nd ed. B. N. Fields and D. M. Knipe (ed.), Raven Press, Ltd., New York.
5. **Althage, A., B. Odermatt, T. Moskophidis, T. Kundig, U. Hoffman-Rohrer, H. Hengartner, and R. M. Zinkernagel.** 1992. Immunosuppression by lymphocytic choriomeningitis virus infection: competent effector T and B cells but impaired antigen presentation. Eur. J. Immunol. **22:**1803–1812.
6. **Armstrong, J. A., and R. Horne.** 1984. Follicular dendritic cells and virus-like particles in AIDS-related lymphadenopathy. Lancet **ii:**370–372.
7. **Austyn, J. M., and S. Gordon.** 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. **11:**805–815.
8. **Beckford, A. P., R. O. C. Kaschula, and C. Stephen.** 1985. Factors associated with fatal cases of measles: a retrospective autopsy study. S. Afr. Med. J. **68:**858–863.

8a. **Borrow, P.** Unpublished observation.

8b. **Borrow, P., and M. B. A. Oldstone.** Unpublished observations.

9. **Borrow, P., and M. B. A. Oldstone.** 1992. Characterization of lymphocytic choriomeningitis virus-binding protein(s): a candidate cellular receptor for the virus. J. Virol. **66:**7270–7281.
10. **Borrow, P., and M. B. A. Oldstone.** Measles virus: mononuclear cell interactions. Curr. Top. Microbiol. Immunol., in press.
11. **Borrow, P., A. Tishon, and M. B. Oldstone.** 1991. Infection of lymphocytes by a virus that aborts cytotoxic T lymphocyte activity and establishes persistent infection. J. Exp. Med. **174:**203–212.
12. **Breel, M., R. E. Mebius, and G. Kraal.** 1987. Dendritic cells of the mouse recognized by two monoclonal antibodies. Eur. J. Immunol. **17:**1555–1559.
13. **Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent.** 1981. A monoclonal antibody discriminating between subsets of T and B cells. J. Immunol. **127:**2496–2501.
14. **Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. A. Oldstone.** 1980. The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv. Immunol. **30:**275–331.
15. **Byrne, J. A., and M. B. A. Oldstone.** 1984. Biology of cloned cytotoxic T lymphocytes specific for LCMV. Clearance of virus in vivo. J. Virol. **51:**682–686.
16. **Children's Vaccine Initiative.** 1993. Measles control: resetting the agenda. Report of the CVI's ad hoc committee on an investment strategy for measles control. Bellagio, Italy.
17. **Clerici, M., A. L. Landay, H. A. Kessler, R. A. Zajac, R. N. Boswell, S. C. Muluk, and G. M. Shearer.** 1991. Multiple patterns of alloantigen presenting/stimulating cell dysfunction in patients with AIDS. J. Immunol. **146:**2207–2213.
18. **Cobbold, S. P., A. Jayasuriya, A. A. Nash, T. D. Prospero, and H. Waldmann.** 1984. Therapy with monoclonal antibodies by elimination of T cell subsets *in vivo*. Nature (London) **312:**548–551.
19. **Cohen, J.** 1993. What causes the immune system collapse seen in AIDS? Science **260:**1256.
20. **Crocker, P., and S. Gordon.** 1989. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. J. Exp. Med. **169:**1333–1346.
21. **Dutko, F. J., and M. B. A. Oldstone.** 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. J. Gen. Virol. **64:**1689–1698.
22. **Fung-Leung, W. P., T. M. Kundig, R. M. Zinkernagel, and T. W. Mak.** 1991. Immune response against lymphocytic choriomeningitis virus infection in

mice without CD8 expression. J. Exp. Med. **174**:1425–1429.
23. **Gordon, S., P. R. Crocker, S. H. Lee, L. Morris, and S. Rabinowitz.** 1986. *In* R. M. Steinman and R. J. North (ed.), Host-resistance mechanisms to infectious agents, tumors and allografts, p. 121–137. Rockefeller University Press, New York.
24. **Helbert, M. R., J. Làge-Stehr, and N. A. Mitchison.** 1993. Antigen presentation, loss of immunological memory, and AIDS. Immunol. Today **14**:340–344.
25. **Hengel, H., M. Lindner, H. Wagner, and K. Heeg.** 1987. Frequency of herpes simplex virus-specific murine cytotoxic T lymphocyte precursors in mitogen- and antigen-driven primary *in vitro* T cell responses. J. Immunol. **139**:4196–4202.
26. **Horohov, D. W., and B. T. Rouse.** 1986. Virus-induced immunosuppression. Vet. Clin. North Am. Small Anim. Pract. **16**:1097–1127.
27. **Inaba, K., and R. M. Steinman.** 1984. Resting and sensitized T lymphocytes exhibit stimulatory (antigen presenting cell) requirements for growth and lymphocytic release. J. Exp. Med. **160**:1717–1735.
28. **King, C. C., B. D. Jamieson, K. Reddy, N. Bali, R. J. Concepcion, and R. Ahmed.** 1992. Viral infection of the thymus. J. Virol. **66**:3155–3160.
29. **Kohler, M., B. Rüttner, S. Cooper, H. Hengartner, and R. M. Zinkernagel.** 1990. Enhanced tumor susceptibility of immunocompetent mice infected with lymphocytic choriomeningitis virus. Cancer Immunol. Immunother. **32**: 117–124.
30. **Kraal, G., M. Breel, M. Janse, and G. Bruin.** 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J. Exp. Med. **163**:981–987.
31. **Kraal, G., M. Rep, and M. Janse.** 1987. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. Scand. J. Immunol. **26**:653–661.
32. **Langhoff, E., and R. M. Steinman.** 1989. Clonal expansion of human T lymphocytes initiated by dendritic cells. J. Exp. Med. **169**:315–320.
33. **Leist, T. P., E. Rüedi, and R. M. Zinkernagel.** 1988. Virus-triggered immune suppression in mice caused by virus-specific cytotoxic T cells. J. Exp. Med. **167**:1749–1754.
34. **Lo, D., C. R. Reilly, B. Scott, R. Liblau, H. O. McDevitt, and L. C. Burkly.** 1993. Antigen-presenting cells in adoptively transferred and spontaneous autoimmune diabetes. Eur. J. Immunol. **23**:1693–1698.
35. **Macatonia, S. E., M. Gompels, A. J. Pinching, S. Patterson, and S. C. Knight.** 1992. Antigen presentation by macrophages but not by dendritic cells in human immunodeficiency virus (HIV) infection. Immunology **75**: 576–581.
36. **Macatonia, S. E., R. Lau, S. Patterson, A. J. Pinching, and S. C. Knight.** 1990. Dendritic cell infection, depletion and dysfunction in HIV-infected individuals. Immunology **71**:38–45.
37. **Matloubian, M., S. R. Kolhekar, T. Somasundaram, and R. Ahmed.** 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. J. Virol. **67**:7340–7349.
38. **McChesney, M. B., and M. B. A. Oldstone.** 1989. Virus-induced immunosuppression: infections with measles virus and human immunodeficiency virus. Adv. Immunol. **45**:335–380.
39. **Merson, M. H.** 1993. Slowing the spread of HIV: agenda for the 1990s. Science **260**:1266–1268.
40. **Metlay, J. P., M. D. Witmer-Pack, R. Agger, M. T. Crowley, D. Lawless, and R. M. Steinman.** 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J. Exp. Med. **171**:1753–1771.
41. **Mims, C. A.** 1986. Interactions of viruses with the immune system. Clin. Exp. Immunol. **66**:1–16.
42. **Moskophidis, D., H. Pircher, I. Ciernik, B. Odermatt, H. Hengartner, and R. M. Zinkernagel.** 1992. Suppression of virus-specific antibody production by $CD8^+$ class I-restricted antiviral cytotoxic T cells in vivo. J. Virol. **66**: 3661–3668.
43. **Murphy, F. A., M. J. Buchmeier, and W. E. Rawls.** 1977. The reticuloendothelium as the target in a virus infection. Pichinde virus pathogenesis in two strains of hamsters. Lab. Invest. **37**:502–515.
44. **Nussenzweig, M. C., R. M. Steinman, M. D. Witmer, and B. Gutchinov.** 1982. A monoclonal antibody specific for mouse dendritic cells. Proc. Natl. Acad. Sci. USA **79**:161–165.
45. **Odermatt, B., M. Eppler, T. P. Leist, H. Hengartner, and R. M. Zinkernagel.** 1991. Virus-triggered acquired immunodeficiency by cytotoxic T-cell-dependent destruction of antigen-presenting cells and lymph follicle structure. Proc. Natl. Acad. Sci. USA **88**:8252–8256.
46. **Oldstone, M. B. A.** 1987. Immunotherapy for virus infection. Curr. Top. Microbiol. Immunol. **134**:211–229.
47. **Oldstone, M. B. A.** 1989. Viral persistence. Cell **56**:517–520.
48. **Oldstone, M. B. A.** 1991. Molecular anatomy of viral persistence. J. Virol. **65**:6381–6386.
49. **Oldstone, M. B. A., A. Tishon, J. M. Chiller, W. O. Weigle, and F. J. Dixon.** 1973. Effect of chronic viral infection on the immune system. I. Comparison of the immune responsiveness of mice chronically infected with LCM virus with that of noninfected mice. J. Immunol. **110**:1268–1278.
50. **Ozato, K., and D. H. Sachs.** 1981. Monoclonal antibodies to mouse MHC antigens. III. Hybridoma antibodies reacting to antigens of the $H\text{-}2^b$ haplotype reveal genetic control of isotype expression. J. Immunol. **126**:317–321.
51. **Parekh, B. S., and M. J. Buchmeier.** 1986. Proteins of LCMV: antigenic topography of the viral glycoproteins. Virology **153**:168–178.
52. **Pircher, H., K. Burki, R. Lang, H. Hengartner, and R. M. Zinkernagel.** 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature (London) **342**:559–561.
53. **Riviere, Y., R. Ahmed, P. J. Southern, M. J. Buchmeier, F. J. Dutko, and M. B. A. Oldstone.** 1985. The S RNA segment of lymphocytic choriomeningitis virus codes for the nucleoprotein and glycoproteins 1 and 2. J. Virol. **53**:966–968.
54. **Roost, H., S. Charan, R. Gobet, E. Ruedi, H. Hengartner, A. Althage, and R. M. Zinkernagel.** 1988. An acquired immune suppression in mice caused by infection with LCMV. Eur. J. Immunol. **4**:511–518.
55. **Rüedi, E., H. Hengartner, and R. M. Zinkernagel.** 1990. Immunosuppression in mice by lymphocytic choriomeningitis virus infection: time dependence during primary and absence of effects on secondary antibody response. Cell. Immunol. **130**:501–512.
56. **Salvato, M., P. Borrow, E. Shimomaye, and M. B. A. Oldstone.** 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. J. Virol. **65**:1863–1869.
57. **Salvato, M. S., and E. M. Shimomaye.** 1989. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. Virology **173**:1–10.
58. **Salvato, M., E. Shimomaye, P. Southern, and M. B. A. Oldstone.** 1988. Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL^+) small genomic segment and that of its variant, clone 13 (CTL^-). Virology **104**:517–522.
59. **Southern, P. J., M. K. Singh, Y. Riviere, D. R. Jacoby, M. J. Buchmeier, and M. B. A. Oldstone.** 1987. Molecular characterization of the genomic S RNA segment from lymphocytic choriomeningitis virus. Virology **157**:145–155.
60. **Sprent, J., and M. Schaefer.** 1985. Properties of purified T cell subsets. I. *In vitro* responses to class I vs. class II H-2 alloantigens. J. Exp. Med. **162**:2068–2088.
61. **Steinman, R. M.** 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. **9**:271–296.
62. **Swiggard, W. J., R. M. Nonacs, M. D. Witmer-Pack, and R. M. Steinman.** 1992. Enrichment of dendritic cells by plastic adherence and EA rosetting, p. 3.7.1–3.7.11. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology, vol. 2. Greene Publishing Associates and Wiley-Interscience, New York.
63. **Tenner-Racz, K., P. Racz, M. Dietrich, and P. Kern.** 1985. Altered follicular dendritic cells and virus-like particles in AIDS and AIDS-related lymphadenopathy. Lancet **i**:105–106.
64. **Tishon, A., P. Borrow, C. Evans, and M. B. Oldstone.** 1993. Virus-induced immunosuppression. I. Age at infection relates to a selective or generalized defect. Virology **195**:397–405.
65. **Whittle, H. C., and W. C. Marshall.** 1987. Measles, p. 5.89–5.93. *In* D. J. Weatherall, J. G. G. Ledingham, and D. A. Warrell (ed.), Oxford textbook of medicine, 2nd ed. Oxford University Press, Oxford.
66. **Wilson, M. C., and G. A. Higgins.** 1989. In situ hybridization, p. 239–284. *In* A. A. Goultong, G. B. Baker, and A. T. Campagnoni (ed.), Neuromethods, vol. 16. Molecular neurobiological techniques. Humana Press, Clifton, N.J.
67. **Wright, K. E., and M. J. Buchmeier.** 1991. Antiviral antibodies attenuate T-cell-mediated immunopathology following acute lymphocytic choriomeningitis virus infection. J. Virol. **65**:3001–3006.
68. **Wu-Hsieh, B., D. H. Howard, and R. Ahmed.** 1988. Virus-induced immunosuppression: a murine model of susceptibility of opportunistic infection. J. Infect. Dis. **158**:232–235.